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FACTORS THAT AFFECT FLUORESCEIN ANALYSIS

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Quality assurance aspects are of considerable consequence in experimental studies. Because of the widespread use of fluorescein as an analytical tracer in aerosol studies, a set of experiments was conducted that summarize the effects of parameters that influence its detection and recovery from filters. The results confirmed the following: (1) the optimum excitation and emission wavelengths for fluorescein are 492 and 516 nm, respectively; (2) the fluorescence intensity from a fluorescein solution is strongly pH dependent; (3) pH does not affect the shape or the position of the peak of the emission spectrum; (4) changes in solution temperature cause a change in fluorescent intensity; (5) at concentrations below the self-quenching limit, fluorometer readings increase in direct proportion to fluorescein concentration; (6) the recovery procedure of fluorescein from glass fiber filters is quantitative; (7) fluorescein samples, prepared for fluorometer measurements, appear to be stable over at least a 12-day period; and (8) at higher gain settings, use of different test tubes and changes in test tube orientation can affect the fluorescence intensity measurement of low fluorescence material. As an example, the procedures are applied to determine aerosol penetration through two sample transport lines.

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PREFACE

The work described in this report was authorized under Project No. 622384/ACB2, Non-Medical CB Defense, and Contract No. DAAD13-98-M-0083. The work was started in November 1998 and completed in May 2001.

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FACTORS THAT AFFECT FLUORESCEIN ANALYSIS

1. INTRODUCTION

Fluorescence is exhibited by organic compounds that have conjugated double bonds (alternating single and double bonds). These compounds emit longer wavelength light when excited by a radiation source of appropriate shorter wavelength (higher energy) light. The emitted light is of longer wavelength because energy is lost in the internal transitions of the molecule. Provided quenching is minimal (e.g., low concentrations in solution), the intensity of emitted light is proportional to the concentration of the compound.

There are three principal types of fluorescein used in aerosol studies: fluorescein, sodium fluorescein (uranine), and ammonium fluorescein (Stober and Flachsbart)[1]. Uranine particles are hygroscopic while ammonium fluorescein particles are not. However, hygroscopicity is of little consequence when fluorescein is used as a tracer in oil droplets, which is the most common approach in contemporary aerosol studies. Fluorescein finds widespread application in aerosol studies because it has a large fluorescent cross section, which allows it to be detected at low concentrations; it can be used as a tracer in liquid droplets or as solid particles; and, it can easily be recovered from locations where it is deposited.

Robinson et al.[2] reported on the use of fluorescein-tagged aerosol as a tracer in meteorological studies. They claimed a minimum detectable uranine concentration of 10^{-11} g/mL. In contrast, Stein et al.[3] reported the fluorescence technique offers a limiting detectability of 10^{-10} g/mL. Burgess et al.[4] used uranine aerosol to test respirator performance and following this, fluorescein was adapted by the Bureau of Mines for evaluation of respirator performance (Ferber[5]). Schulz et al.[6] used uranine to measure stack emissions. Stein et al.[3] measured the density of uranine aerosol particles so that they could be used to calibrate an aerosol spectrometer. Stober and Flachsbart[1] evaluated ammonium fluorescein as a laboratory test aerosol. The US Environmental Protection Agency[7] employs fluorescein aerosols in standard methodology to evaluate the performance of ambient samplers; and, McFarland et al.[8] used fluorescein to characterize the performance of stack sampling systems.

There are many factors that affect fluorometry including intensity and wavelength of primary light and bandwidth of excitation and emission filters used in the fluorometer. Fluorescence can be disturbed by changes in the environment (e.g., the pH, ionic state of the molecule, nature of the solvent, degree of subdivision of the material, viscosity, temperature, and the introduction of certain chemical groups, Van Duuren[9]). Thus, experimental conditions should be meticulously controlled in quantitative fluorescence studies. Disadvantages of fluorometry are that it is non-specific and the fluorescence of many compounds fade very rapidly (Lodge[10]). Also, quenching (self-quenching) can be observed in luminescence processes – if the temperature decreases, or there are increases in the amount of oxygen in the solution, concentration of the fluorescing material, and the amount of impurities, fluorescence can decrease due to quenching (Guilbault[11]).

In our experimental testing, fluorescein tagged aerosols are usually collected by sampling on filters. The fluorescein is then eluted with a solvent and a fluorometer is used to measure the concentration of fluorescein in the solution. Light from a UV source excites the solution, which is contained in a glass test tube, and the fluoresced light emitted by the sample is measured with a high-gain, low-noise photomultiplier tube. A narrow band optical filter is placed between the light source and the sample, and a sharp cut (high pass), optical filter is used to minimize the amount of short wavelength radiation that can be transmitted to the photomultiplier tube. The optical filters must be optimized to allow transmission of the excitation peak through the narrow band optical filter and transmission of the

emission peak through the high pass filter. For work with fluorescein, we use optical filters supplied by Barnstead/Thermolyne (Dubuque, IA); in particular, an NB490 narrow band filter, which has a peak transmission at 490 nm and a nominal band width of 10 nm, and an SC515 sharp cut filter, which has a transmission of about 37% at a wavelength of 515 nm and less than 1% at 505 nm.

This study, which is focused on quality assurance aspects of the use of fluorescein in aerosol studies, has two parts. The first involves examining factors that affect the analysis and recovery of fluorescein, and the second part involves application of the procedures by experimentally determining the penetration of aerosol through two aerosol tubes and evaluating the precision of the methodology.

2. EXPERIMENTAL METHODS AND RESULTS

2.1 Part I: Detection and Recovery of Fluorescein.

2.1.1 Optimum Excitation and Emission Wavelengths for Fluorescein.

Tests were conducted to measure the optimum excitation wavelength of a fluorescein sample (Chemical Abstracts Registry No. CAS-518-47-8, Acros Organics, Morris Plains, NJ) and its corresponding emission spectrum. For most materials the optimum excitation wavelength is equal to the highest UV absorption wavelength. In this study, a UV absorption spectrum was obtained using a spectrophotometer (Model DU 7400, Beckman Instruments, Inc., Fullerton, CA). The wavelength with the highest UV absorption was then used to excite a fluorescein sample in a spectrofluorometer (Spex Fluorolog-2, Spex Industries, Inc., Edison, NJ) to obtain the emission spectrum. To confirm the optimum excitation wavelength obtained from the UV absorption spectrum, the magnitude of the emission peak was recorded for a range of excitation wavelengths using the spectrofluorometer. In addition, the spectrofluorometer was used to measure emission spectra for a range of excitation wavelengths to construct a 3-dimensional graph of fluorescence intensity as a function of excitation and emission wavelengths.

Figure 1 shows excitation and emission spectra, where it may be observed that the peak excitation wavelength is 492 nm and the emission peak is 516 nm. Ward and Fothergill[12] conducted similar experiments and reported that the absorption peak of fluorescein is 490 nm and the emission peak is 520 nm. The results in Figure 1 suggest that the excitation optical filter of a fluorometer should allow transmission of 492 nm light and the emission optical filter (sharp cut, high pass) should allow passage of 516 nm light. Also, there should be no overlapping of the transmission characteristics. These requirements are fulfilled by use of the NB490 and SC515 filters.

Figure 2 shows emission fluorescence intensity as a function of excitation and emission wavelengths. From this 3-dimensional graph, it may be noted that for fluorescein samples, there is a clearly defined optimum emission wavelength (at about 517 nm in this graph) for any given excitation wavelength. The wavelength for peak excitation on the 3-D graph is about 496 nm versus the value of 492 nm obtained from the database for the results shown in Figure 1. The reason for the differences between the results in Figures 1 and 2 is that more effort was devoted to delineating the peaks in the data for Figure 1; therefore, those results should be used for the values of the peak wavelengths.

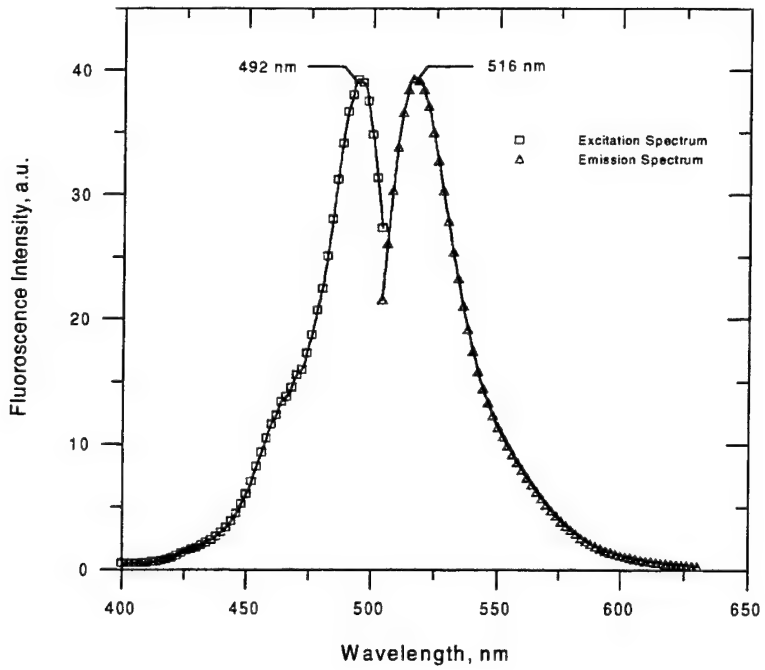

Figure 1. Excitation and Emission Spectra of Fluorescein.

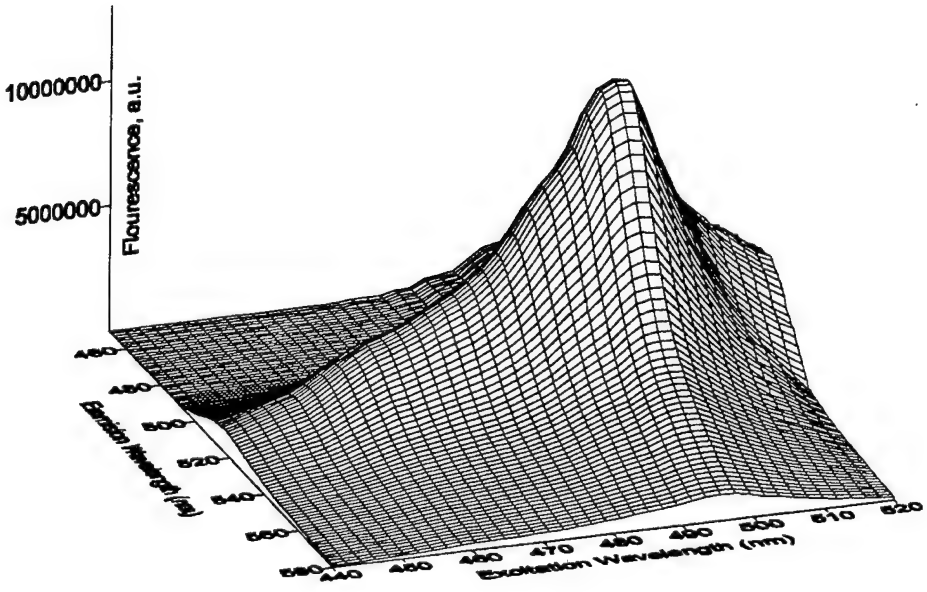

Figure 2. 3-D Plot of Fluorescence Intensity as a Function of Excitation and Emission Wavelengths. The emission spectrum peaks at about 516 μm independent of excitation wavelength.

2.1.2 Effect of Solution pH on Fluorescence Intensity.

This experiment was conducted to quantify the effects of the pH of a fluorescein solution on the observed fluorescence intensity. The range of pH used in these experiments was 2 to 10. Hydrochloric acid was used to lower the pH of the solution and then small amounts of ammonium hydroxide were added to increase the pH. Readings were corrected for the small increase in volume due to the addition of base to adjust the pH.

Figure 3 shows fluorescence as a function of the pH of the solution. Fluorescence is near zero when the pH is low (acidic) and the readings increase and ultimately plateau between a pH of 8 and 10. Ward and Fothergill[12] had previously conducted experiments to characterize fluorescence as a function of pH, and reported that fluorescence is 50% lower at a pH of 6 than at a pH of 8.

In our test protocol with fluorescein aerosols, we use a solution of deionized/distilled water and 2-propanol (50/50 on a v/v basis) to which is added 0.056% of 14.8 N ammonium hydroxide. This solution, which will be referred to herein as the recovery solution, has a pH of 9.8.

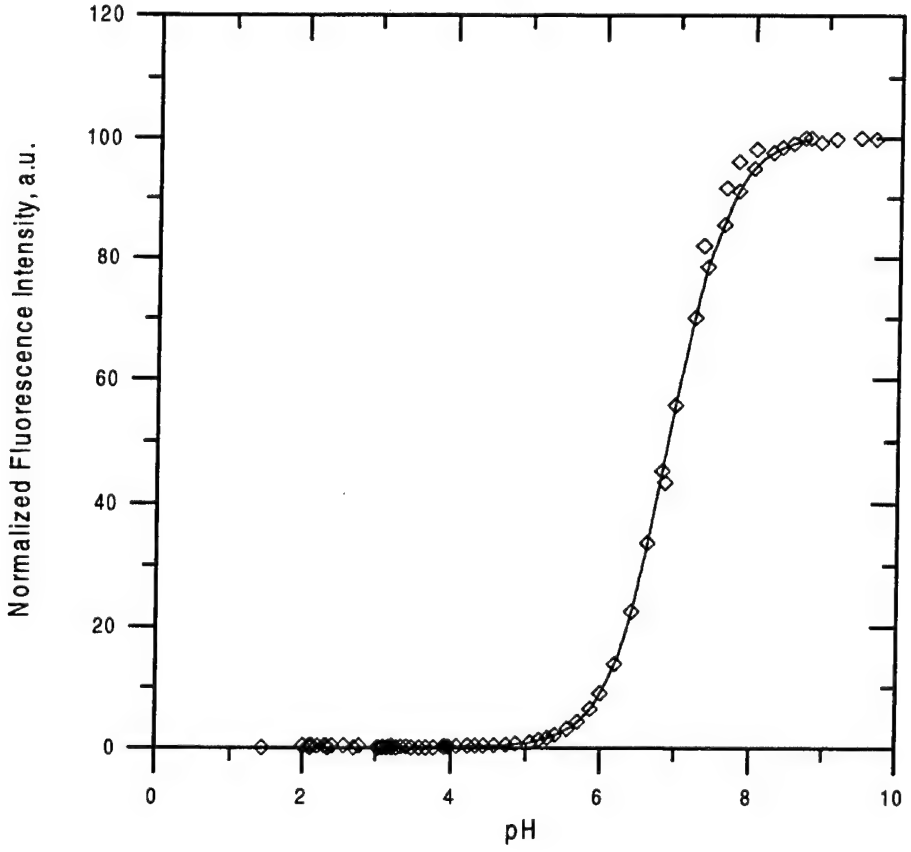

Figure 3. Normalized Fluorescence Intensity of Fluorescein as a Function of the Solution.

2.1.3 Effect of Solution pH on Shape and Wavelength of the Emission Peak.

The emission spectra of fluorescein solutions was checked with pH values (7 and 9.8) to determine if pH would affect the emission curve. If there were to be a shift, then pH could confound test results not only by affecting the intensity, but also by affecting the relative amount of light that would be transmitted through the high pass optical emission filter.

The samples were excited with a wavelength of 492 nm and the emission spectra were measured with the spectrofluorometer. Data, which were normalized to the highest readings, are shown in Figure 4 where it may be noted the two emission spectra are identical. This information allows researchers to have confidence that small changes in pH will not affect the readings because of spectrum shifts.

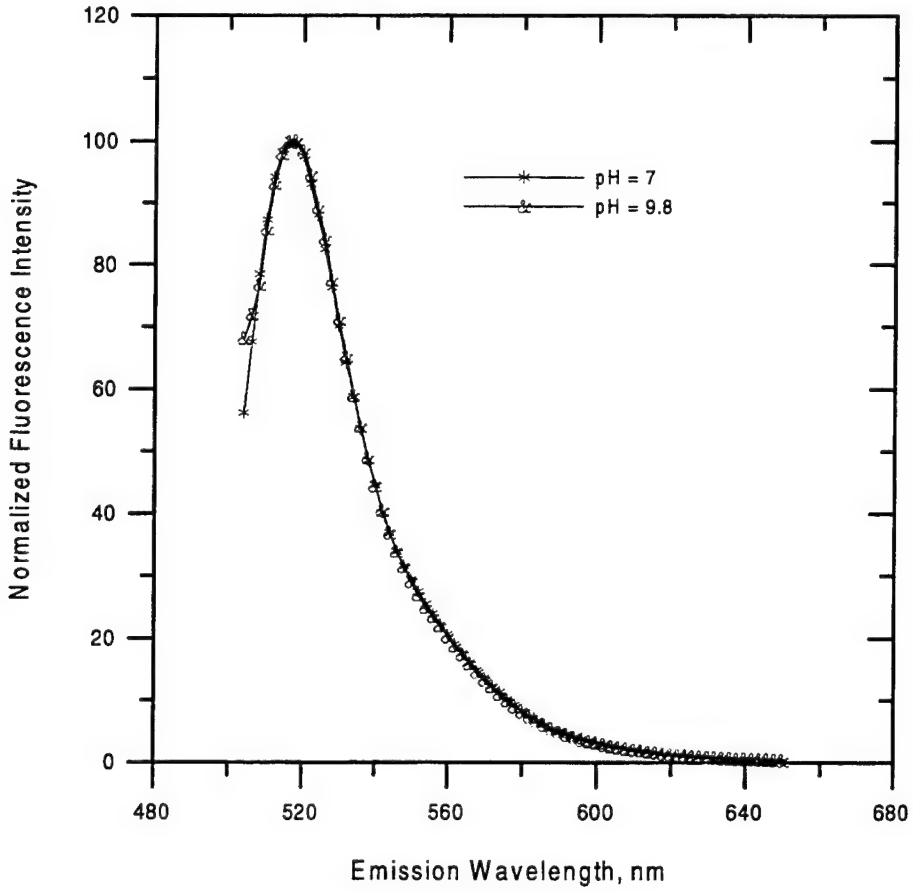

Figure 4. Emission Spectra of Solutions with pH Values of 7 and 9.8. There is no change in spectra with respect to pH over this range of values.

2.1.4 Effect of Temperature on Fluorometer Readings.

Six mixtures of approximately 3×10^{-9} g/mL of fluorescein in the recovery solution were tested over a range of temperatures from about 8°C to 38°C to determine the effect of temperature on reading. The mixtures were heated or cooled in test tubes in a water bath until thermal equilibrium was

reached and then immediately placed in the fluorometer. There was less than 1°C change in the temperature of a sample from the time it was removed from the bath until the reading was completed. The results of the experiments, Figure 5, show there is a decrease in fluorometer reading as temperature increases. If it is assumed the relationship between reading and temperature is linear over the range of test temperatures, then based on linear regressions of the six data sets shown in Figure 5, the average change in the normalized reading is –0.8%/°C, i.e.,:

$$\frac{dR/R_0}{dT} = -0.8\ \%/°C \tag{1}$$

where: R = fluorometer reading; R_o = reading at a temperature of 0°C (intercept of the regression line); and T = temperature in units of °C.

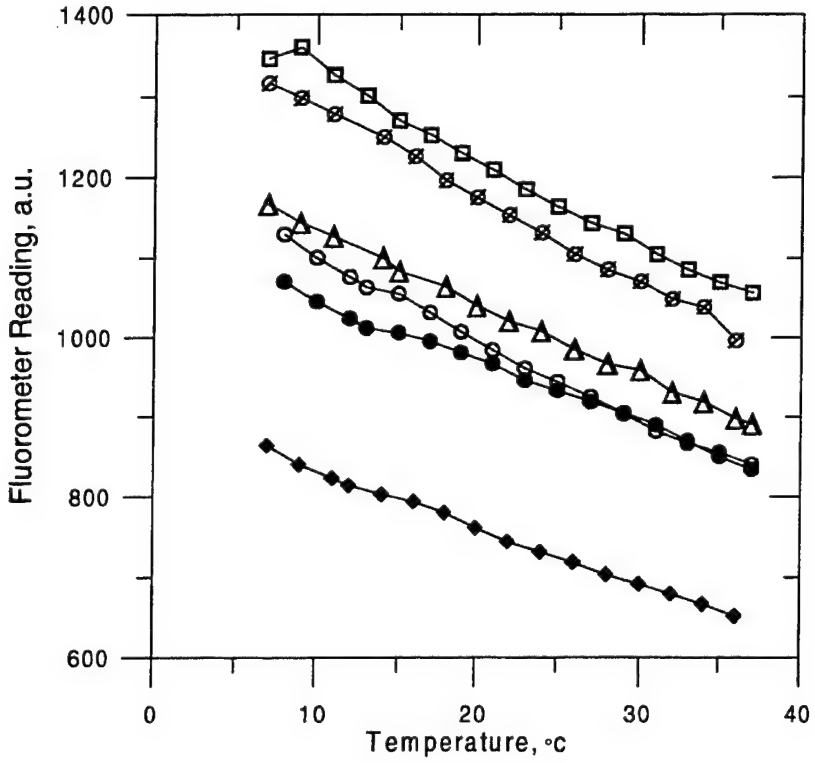

Figure 5. Effect of Temperature on Fluorometer Readings. The change in readings averaged over all six runs is 0.76 %/°C.

2.1.5 Proportionality of Fluorescein Concentration and Fluorometer Reading.

Experiments were conducted to test the proportionality of fluorometer readings and fluorescein concentration. The relationship between concentration, c, and fluorometer reading, R, can be expressed as,

$$R = a\,c^b \tag{2}$$

where: a and b are constants. If R and c are directly proportional, the exponent b will be unity.

The fluorometer used in the tests has scale factors of 1X to 1000X, which are achieved by changing the size of an aperture between the excitation filter and the sample. Proportionality tests for readings that occur on multiple scales could be used to check the scale factors.

Solutions containing weighed quantities of fluorescein in measured volumes of liquid were prepared, serially diluted and then measured fluorometrically. Concentrations spanned the ranges covered by the 1X, 10X and 50X scales of the Sequoia Turner Model 450 Fluorometer, which are the scales that are almost exclusively utilized in our applications. The maximum reading of the fluorometer, which is 2000 on the 1X scale, corresponds to a concentration of about 1.5 µg/mL of fluorescein.

Figure 6 shows fluorometer readings as functions of fluorescein concentration with lines drawn through the data that are regression fits to the logarithm of Equation 2, i.e.,:

$$\ln R = \ln a + b \ln c \tag{3}$$

For the regression lines, the units of R are the arbitrary (those from the instrument reading) and the units of c are fluorescein concentration (g/mL). The crucial consideration is whether the values of b are unity, for if they are, the ratio of fluorescein concentration in two samples can be determined by the ratio of the instrument readings provided both readings are on the same scale. With reference to Table 1, values of b and the 95% confidence limits for b, are given for the three instrument scales. The 95% confidence intervals of b for the 10X and 50X scales clearly overlap unity, while the full data set for the 1X scale (readings from 15 to 1998, Table 1) shows a slope of 0.953 with a 95% confidence interval of 0.943 to 0.964. When readings > 1000 (concentrations > 0.75 µg/mL are removed from the data set, the slope of the 1X data set increases to 0.982 and the 95% confidence interval becomes 0.967 to 0.997. The regression line for the 1X data set shown in Figure 6 is based on the truncated data set (readings < 1000).

Table 1. Proportionality of Fluorometer Reading to Fluorescein Concentration. The parameter b is from a regression fit of experimental data to the relationship, $\ln R = \ln a + b \ln c$, where R is the instrument reading and c is the fluorescein concentration (g/mL). A value of unity for b means the reading is directly proportional to the concentration.

Instrument Scale	Range of Readings	No. of Readings	Slope of regression line, b	95% Confidence Interval of b	Standard Error of $\ln R$
1X	15 to 1998	49	0.953	0.943 to 0.964	0.0613
1X	15 to 996	26	0.982	0.967 to 0.997	0.0447
10X	25 to 1406	19	1.018	0.995 to 1.042	0.0624
50X	126 to 1811	30	1.039	0.955 to 1.124	0.120

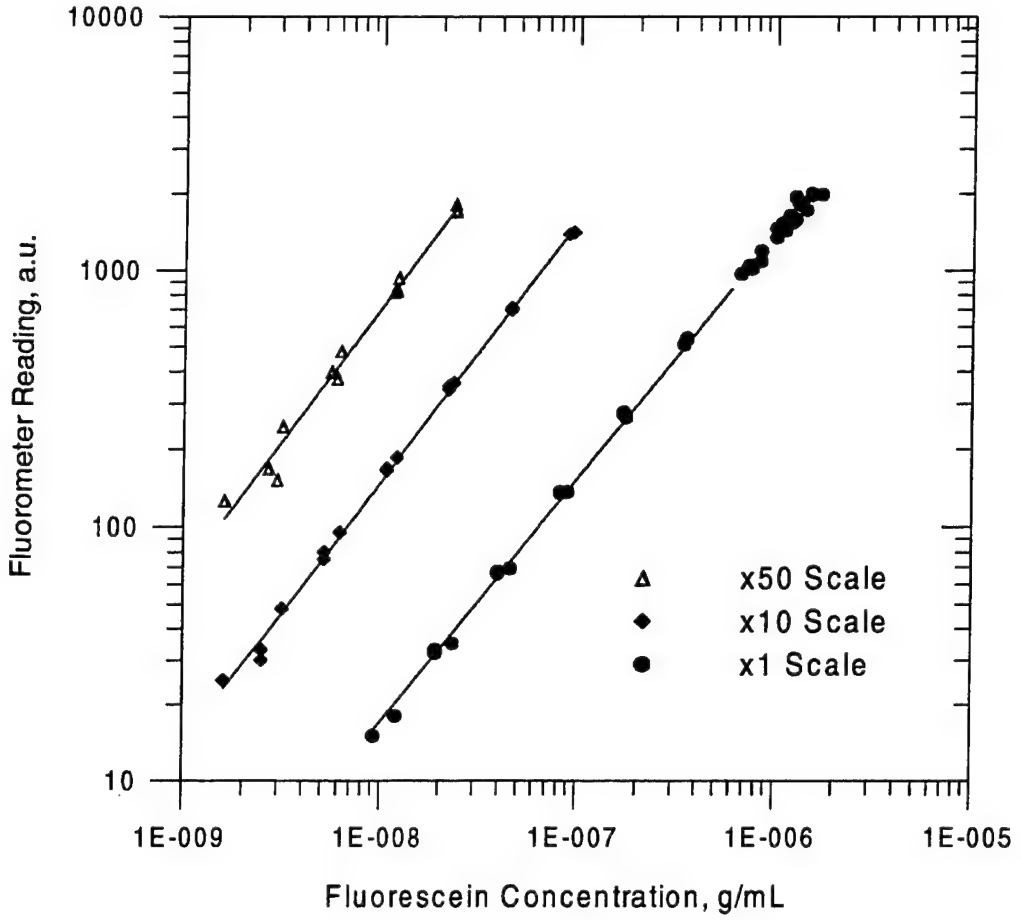

Figure 6. Fluorometer Readings as a Function of Fluorescein Concentration. A slope of unity on a log-log plot indicates the reading is proportional to concentration.

In general, these results support the work of Robinson et al.[2], who showed that the response of an instrument is a linear function of uranine concentration for solutions with concentrations less than about 10^{-6} g/mL and Guilbault[11], who showed that the fluorescence intensity of naphthalene increased linearly with concentration. However, we prefer to place the upper limit on concentration at 0.75 µg/mL rather than 1 µg/mL.

The standard error of the readings about the regression line of Equation 3 was calculated and the results show the standard error of ln *R* to vary from 0.045 for the readings on the 1X scale to 0.12 for readings on the 50X scale. For the 1X scale, when exponentiated, (+) one standard error on *R* is the value of R 1.05 and (–) one standard error will be the value of *R* divided by 1.05. The 95% confidence limits on a fluorometer reading for a given concentration can be estimated through use of the statistical *t*-distribution and the standard error on ln *R* (Milton and Arnold)[13]. At a reading of 500 on the 1X scale, the 95% confidence interval is 500/1.12 to 500×1.12 or 446 to 560. For mid-scale readings (i.e., R = 1000) on the 10X and 50 X scales, the factor of 1.12 for the 1X scale is replaced by 1.17 and 1.34, respectively.

Ratios of the scale factors can be estimated by first assuming a concentration, then using Equation 3 to calculate values of the resulting readings on the selected scales. The results of such calculations are shown in Table 2, where the predicted readings on the 1X, 10X and 50X scales are given as functions of assumed concentration values. Readings less than 10 are excluded from consideration. Ratios of predicted readings are shown in the table, where it may be noted the geometric mean of the 10X/1X ratio data is 9.48, and that of the 50X/10X is 4.70. Because the slopes of the regression curves are not unity, the scale ratio estimates, as shown in Table 2, vary somewhat with concentration.

Table 2. Predicted Scale Ratios from Use of Regression Equations. Readings <10 and >2000 are excluded.

Assumed Concentration, g/L	Predicted Reading on Given Scale from Regression Equations			Ratio of Predicted Readings	
	1X	10X	50X	10X/1X	50X/10X
1.00E-9		14.3	65.3		4.57
2.00E-9		28.9	134.1		4.63
4.00E-9		58.6	275.6		4.70
8.00E-9	13.2	118.8	566	9.01	4.77
1.60E-8	26.0	241	1164	9.24	4.84
3.20E-8	51.4	487		9.48	
6.40E-8	101.5	987		9.73	
1.28E-7	200.4	2000		9.98	
Geometric Mean Values				9.48	4.70

2.1.6 Recovery Fluorescein from Glass Fiber Filters.

This experiment was conducted to confirm that the recovery of fluorescein from sampling filters is efficacious. A measured amount of a mixture of recovery solution and fluorescein was added to six 47-mm Gelman Type A/E filters (Pall Gelman Laboratory, Ann Arbor, MI). The filters were then air-dried and placed individually in test tubes. Twenty mL of recovery solution was added to the test tubes and similarly 20 mL of the mixture of recovery solution and fluorescein was added to each of six test tubes without filters. The test tubes were shaken for 1 hour with a laboratory table rotator (Lab-Line Instruments, Inc., Melrose Park, IL). The fluorescence intensity of each mixture was then measured with the fluorometer and the readings were normalized to the relative amount of fluorescein added to each tube.

Figure 7 shows a comparison of the readings for the mixtures containing fluorescein extracted from the filters with the readings from the original mixture used to spike the filters. The normalized readings from the tubes containing the spiked filters were 576 ± 20 (mean ± 1 standard deviation) and those from the control tubes were 582 ± 44. A statistical *t*-test at the 95% confidence level accepts the hypothesis the two means are equal. This shows that the method used for removing fluorescein from glass fiber filters is quantitative, i.e., total recovery of the fluorescein from filter samples.

The study of Robinson et al.[2] showed that removal of uranine from filters takes only a few minutes. In their procedure, water was added to a filter, the filter remained in the solution for a few minutes, and then the liquid was analyzed with a fluorescence meter.

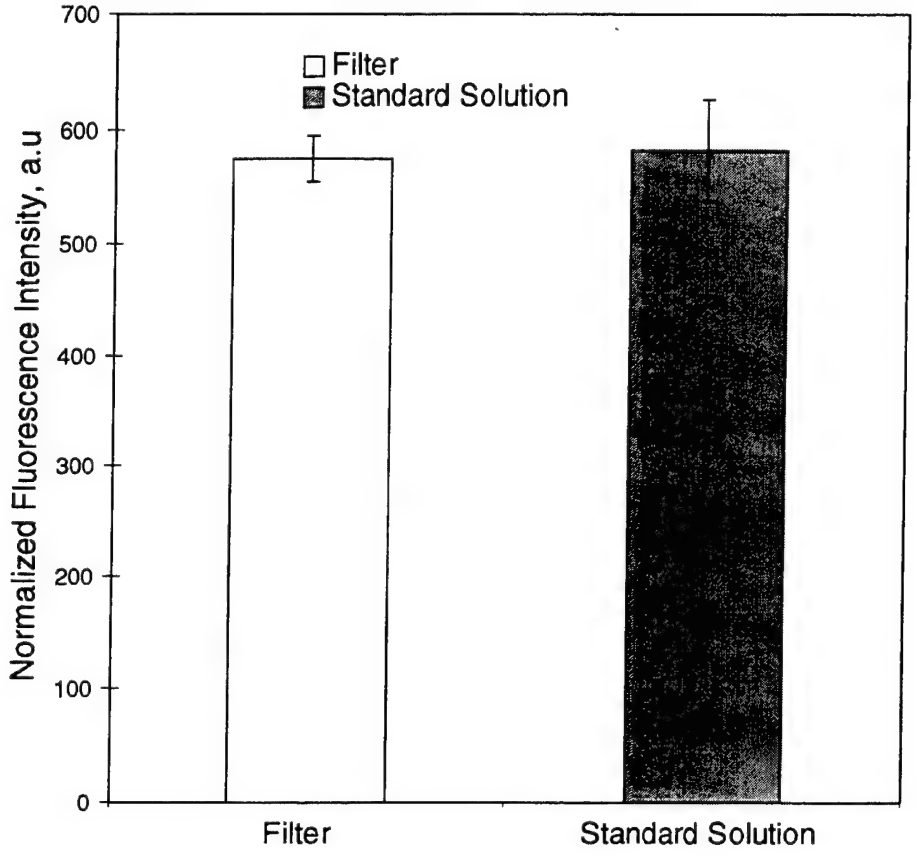

Figure 7. Fluorometer Readings for Samples of Fluorescein Recovered from Filters Compared with Readings for Equal Amounts of Fluorescein Added Directly to Solutions.

2.1.7 Stability of Fluorescein Solutions Over a 12-Day Period

With aerosol experiments, the generation and sampling may take place during one day and the analysis a day later. Guilbault[11] and Lodge[10] state that the fluorescence of many compounds fades rapidly with time. Guilbault reports that in 100 minutes, the fluorescence of a quinine sulfate solution decreases by 32%. To check this effect with fluorescein, an experiment was conducted to determine the stability over a 12-day period. Seven mixtures of fluorescein, which had been extracted from test filters in recovery solution, were stored in tightly covered test tubes on laboratory benches. The fluorescence of

the samples was measured with the fluorometer at various intervals over the 12-day period. The samples were exposed to normal lighting in the laboratory (fluorescent bulbs) during working hours, but the lights were turned off nights and weekends. Figure 8 shows the fluorometer readings of samples over the exposure period, where it may be noted there is no systematic decay in readings. In general, the measurements for a given sample are within ± 2 units of its mean, so if the mean is 50 units, the reproducibility is ± 5% over the 12-day period.

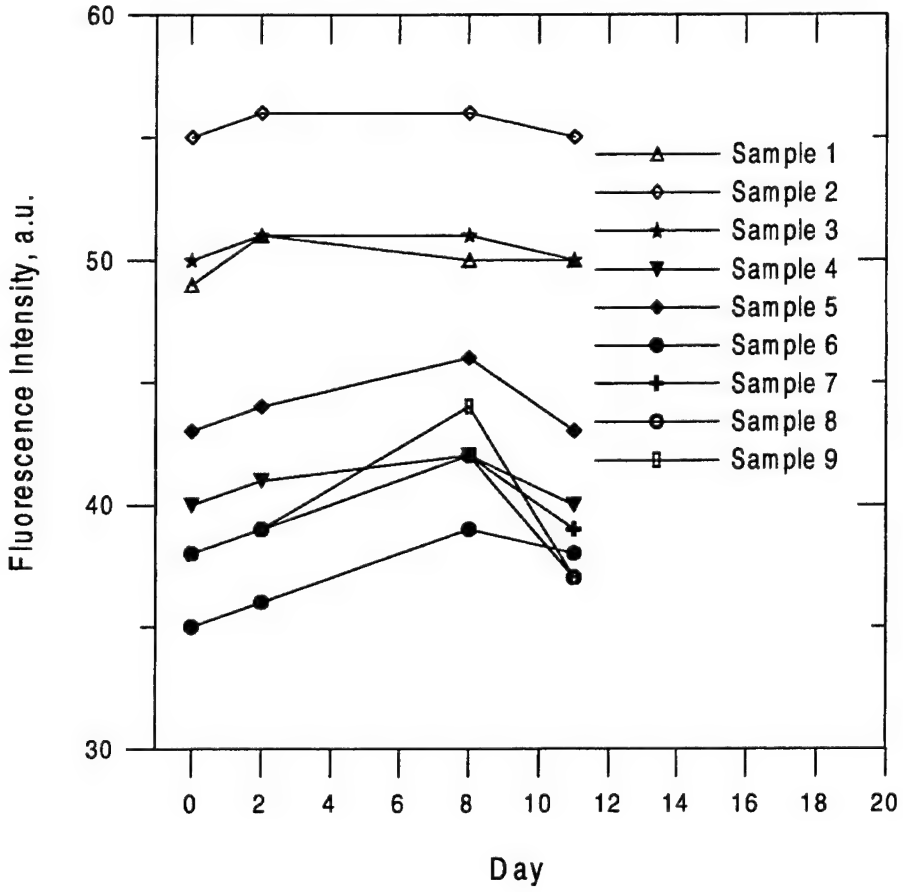

Figure 8. Stability of Fluorescein Over a 12-Day Period.

2.1.8 Use of Different Test Tubes.

The fluorometer operating manual (Barnstead/Thermolyne Corporation[14]) suggests that ordinary borosilicate glass tubes may be used when excitation and emission wavelengths are above 320 nm, and synthetic silica cells are recommended for lower excitation wavelengths. For our experiments, we chose the less expensive borosilicate glass test tubes because both excitation and emission wavelengths are well above 320 nm.

In our laboratory procedure, test tubes are discarded after each use. Therefore, an experiment was conducted to determine the variation in fluorescence intensity measurements due to the use of different test tubes. Six test tubes (12 mm diameter × 75 mm long) were filled with deionized water and fluorescence was measured with the Turner Model 450 at gain settings of 1, 5, 10, 50, 200, and 1000. The test tube with the lowest reading was taken as the reference sample and the fluorometer was

zeroed against that test tube at each gain setting. Fluorescence intensity measurements of the remaining 5 test tubes were recorded. Table 3 shows the measurement mean, standard deviation, and range of the measurements on the five test tubes relative to the zero value of the reference tube.

Table 3. Fluorescence Measurements of Six Test Tubes Filled with Recovery Solution. Test tube size = 12 mm diameter × 75mm long. The reference test tube was selected as the one with the lowest background readings and the fluorometer was zeroed to that tube at each gain (scale) setting.

Scale	Fluorometer reading: mean, ± 1 std. dev. (range)	
	Reference Test Tube	Measurements of six test tubes
1X	0	0 ± 0 (0)
5X	0	0 ± 0 (0)
10X	0	0 ± 0 (0)
50X	0	0.2 ± 0.4 (0-1)
200X	0	3.5 ± 2.9 (0-7)
1000X	0	21.8 ± 14.9 (0-35)

Results show that at gain settings up to 50X, there is less than 1scale unit variation from tube-to-tube. However, at the highest gain setting (1000X), there is a mean reading of 22 ± 15 units relative to the tube with the lowest reading. We do our best to control our experimental variables so the fluorometer readings will be on at least the 50X scale and preferably the 1X scale.

2.1.9 Test Tube Fit and Rotational Alignment.

The effect of test tube fit and orientation in the fluorometer on fluorescence intensity measurements was determined using two different sizes of test tubes. The objective was to determine whether snugly fitting and marked test tubes are needed for fluorometer measurements.

A 10 mm diameter × 75 mm long test tube and a 12×75 mm test tube were filled with deionized/distilled water. For each gain setting, the initial reading was zeroed and the test tube was rotated randomly to a fixed position and fluorometer readings were recorded. At least five random position measurements were recorded at each gain setting. Table 4 shows the range of readings obtained at each gain setting for each test tube.

The 12×75 mm test tube fit snugly in the cuvette holder; therefore, fluorescence measurements had less variation than the 10×75 mm test tube that had an annular space surrounding it, which may allow the tube to be slightly canted. For example, at a gain setting of 200, the values of the mean ± 1 standard deviation were 1.3 ± 1.5 for the 12×75 mm tube, while the values were 27.5 ± 14.5 for the 10×75 mm tube. It is apparent that snugly fitting tubes are needed in order to obtain quality data.

Table 4. Fluorescence Measurements of Two Sizes of Test Tubes at Random Rotational Orientations.

Scale	Fluorometer reading: mean ± 1 std. dev. (range)	
	Test tube size = 10×75 mm	Test tube size = 12×75 mm
1X	0 ± 0 (0)	0 ± 0 (0)
5X	0 ± 0 (0)	0 ± 0 (0)
10X	0 ± 0 (0)	0 ± 0 (0)
50X	2 ± 3.6 (0 – 9)	0.2 ± 0.4 (0 – 1)
200X	27.5 ± 14.5 (0 – 39)	1.3 ± 1.5 (0 – 5)
1000X	41 ± 23.6(0 – 73)	16.3 ± 16.7 (0 – 39)

The fluorometer vendor (Barnstead/Thermolyne Corporation[14]) recommends that when using round cuvettes, the operator align them the same way in the instrument every time. Variation in the glass thickness and properties can be minimized by using this method. To test this recommendation, the 10×75 mm and 12×75 mm test tubes were filled with deionized/distilled water and aligned in the sample chamber during each fluorometer measurement. Ten measurements were taken at each gain setting by inserting the test tube and aligning it in the fluorometer before each measurement. The results, Table 5, show that aligning the 10 × 75 mm test tube in the fluorometer reduces the variation in fluorometer readings as compared with those shown in Table 4; however, alignment of the 12×75 mm tube had little effect. For example, at the gain setting of 200, the mean reading for the randomly oriented 10 × 75 mm tube was 27.5, whereas, the mean reading for the aligned tube was 3.1. By comparison, the mean readings at the 200X gain for the randomly aligned and aligned 12×75 mm tube were 1.3 and 2.1, respectively. This suggests that alignment is not necessary for the snugly fitting 12×75 mm tubes, which in turn implies that use of new tubes for each test is acceptable.

Table 5. Fluorescence Readings (mean ± 1 std. dev. (range)) for Repeated Measurements with Test Tubes Aligned in the Fluorometer. Tubes were filled with recovery solution.

Scale	Tube Size	
	10 mm diameter × 75 mm long	12 mm diameter × 75 mm long
1X	0 ± 0 (0)	0 ± 0 (0)
5X	0.3 ± 0.5 (0 – 1)	0.1 ± 0.3 (0 – 1)
10X	0.4 ± 0.5 (0 – 1)	0.9 ± 0.6 (0 – 2)
50X	2.7 ± 1.5 (0 – 5)	0.8 ± 0.8 (0 – 2)
200X	3.1 ± 3.1 (0 – 11)	2.1 ± 2.6 (0 – 7)
1000X	3.6 ± 3.1 (0 – 10)	11.2 ± 9.2 (0 – 31)

2.1.10 Removal of Fluorescein from Test Tubes During Washing.

Because some laboratories wash (or rinse) and re-use the test tubes, this experiment was conducted to determine how efficiently fluorescein is removed by washing. Three mL of a dilute mixture of 10% fluorescein and 90% oleic acid (mass/volume) in the propanol-water-ammonium hydroxide recovery solution were put into a 10×75 mm test tube and the fluorescence intensity was measured. The original solution was poured out and the tube was washed. The washing was performed by adding 3 mL of the recovery solution to the test tube, closing the open end with Parafilm®, and turning it upside down a few times to remove the fluorescein from the walls of the test tube. Fluorescence intensity measurements of the wash liquid were taken and the wash procedure was repeated until the readings did not decrease with additional washing. The fluorescence intensity measurements were normalized to the reading of the initial solution.

The results, Figure 9, demonstrate that for the washing procedure used herein, three washes reduce the fluorescein concentration by two orders of magnitude, i.e., to about 1% that of the original solution. However, it is recommended that laboratories which re-use test tubes should check the efficacy of the particular wash procedures they employ.

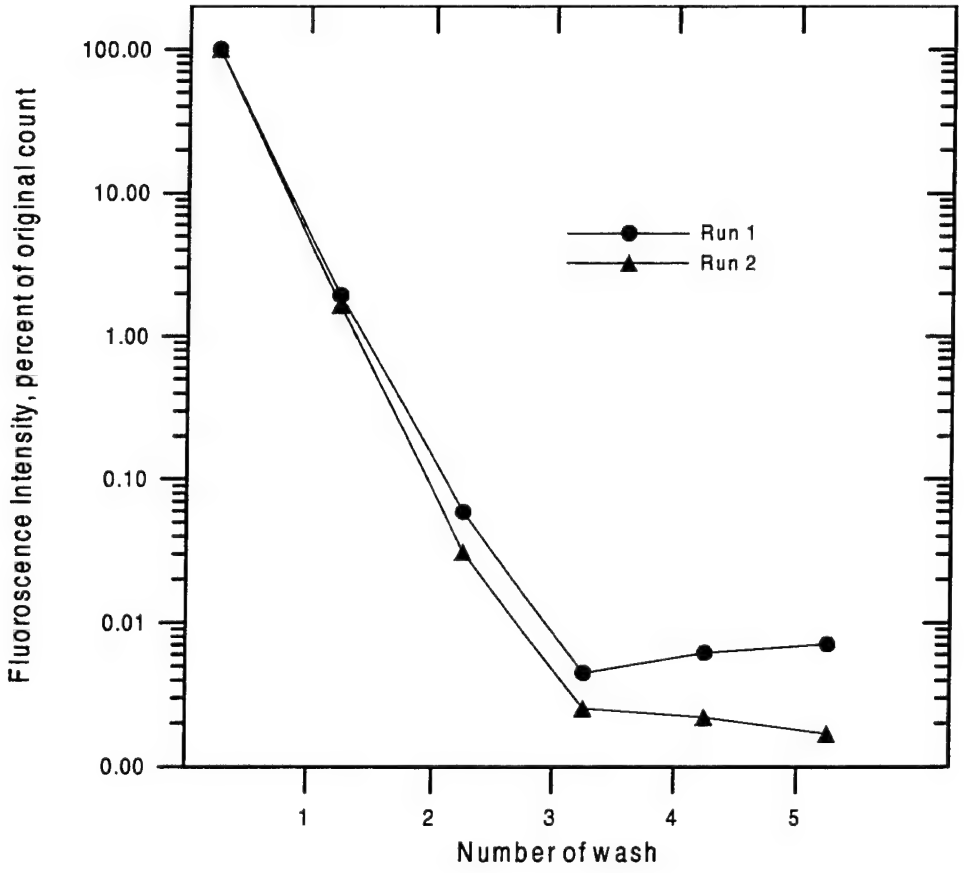

Figure 9. Efficiency of Fluorescein Removal from Test Tubes During Washing.

2.2 Part II: Example of an Application of the Methodology.

2.2.1 Penetration of Aerosol Through Transport Tubes.

An experiment was conducted to determine the penetration of aerosol through two transport tubes using the recommended methods discussed in Part I. This experiment was conducted using monodisperse oleic acid aerosols of 5, 11, and 15 μm aerodynamic diameter that were tagged with fluorescein (90% oleic acid and 10% fluorescein, m/v). Particles were generated with a vibrating orifice aerosol generator (VOAG-Model 3450, TSI Incorporated, St Paul, MN) mounted, as shown in Figure 10, on top of a cubic chamber that was 0.76 m on a side (2.5 feet). Particle size was characterized by collecting samples on glass slides coated with a thin layer of an oil-phobic agent (Nyebar Type W Fluid, William F. Nye, Inc., New Bedford, MA) and measuring the collected droplets with an optical microscope. The measured diameter was converted to aerodynamic diameter using the method described by Olan-Figueroa et al.[15]. An Aerosizer Particle Counter (Amherst Process Instruments, Hadley, MA) was used to monitor the aerosol in the chamber during the experiments for assurance that there were no changes in either aerosol size or concentration.

The aerosol in the chamber was continuously stirred with a fan while samples were drawn through a reference filter (Gelman Type A/E) and the transport tubes, which were followed by sampling filters. The shapes of the aerosol transport tubes that were tested are also shown in Figure 10. Tube 1 is 50.8 mm (2 inches) inside diameter and 2.24 m (7.35 ft) long; and, Tube 2 is 63.5 mm (2.5 inches) diameter by 1.80 m (5.92 ft) long. The additional length of Tube 1 relative to Tube 2 is a longer section of the portion of the tube that is inclined 45° to the horizontal in the figure. Flow rate though Tube 1 was 28.3 L/min and that through Tube 2 was 780 L/min.

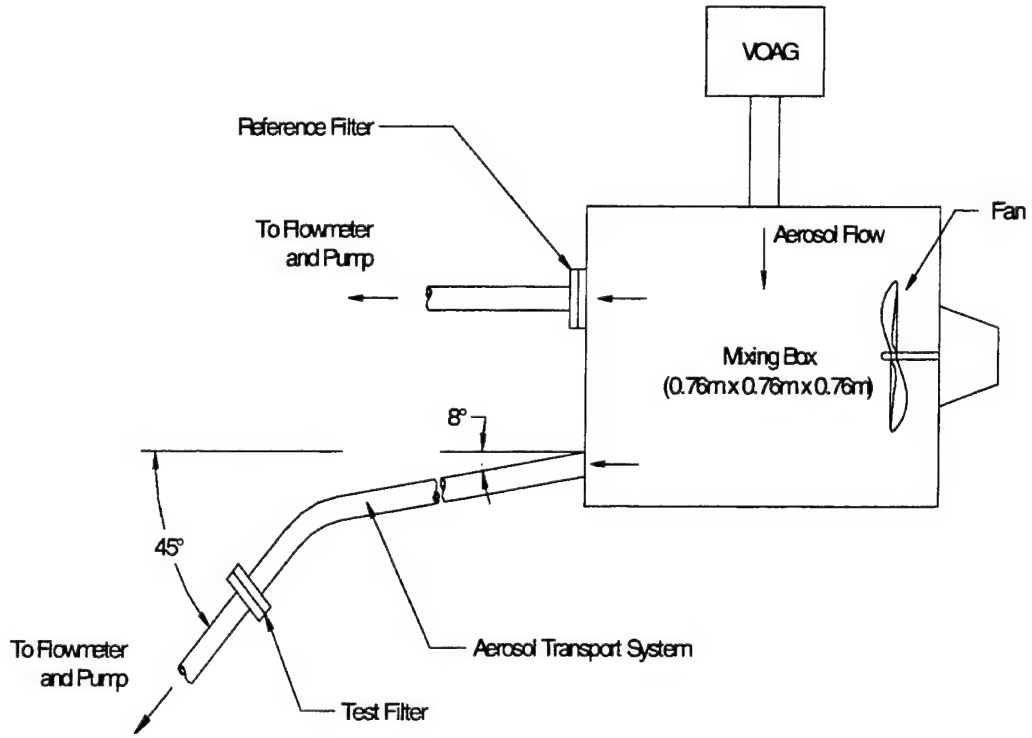

Figure 10. Experimental Setup for Measuring Penetration Through Tubing.

With respect to analysis, the filters were put into measured amounts of recovery solution and were shaken on a table rotator for 1 hour. Aliquots of the solutions were then measured fluorometrically on the 1X scale. Aerosol penetration through the transport tube, P, is calculated from:

$$P = \frac{c_{test}}{c_{ref}} \tag{5}$$

where c_{test} = aerosol concentration in the chamber based on the tracer collected by the filter following the transport tube; and, c_{ref} = aerosol concentration in the chamber based on the aerosol collected by the reference filter. In turn, the aerosol concentration is calculated from,

$$c \propto \frac{R V_L}{q_a t} \tag{6}$$

where R = fluorometer reading; V_L = volume of the solution used for eluting the tracer; q_a = volumetric flow rate of air; and t = sampling time.

The measured aerosol penetration through the tubes is given in Table 6, where it may be noted that at a size of 11 μm aerodynamic diameter (AD), the penetration through Tube 1 was 56% ± 1.0% (mean ± 1 standard deviation) and that through Tube 2 was 96% ± 4.5%. In general terms, because of the way the experiments are conducted and the results are calculated, the standard deviation will increase with penetration.

Table 6. Aerosol Penetration Through Two Aerosol Transport Tubes Shown in Figure 10. Values given are mean penetration ± 1 standard deviation.

Configuration of Transport Tube	Aerodynamic Particle Diameter		
	5 μm	11 μm	15 μm
1[(1)]	98.8% ± 7.3%	56% ± 1.0%	28.8% ± 4.3%
2 [(2)]	90.0% ± 13.2%	96.2% ± 4.5%	

[(1)] Tube 1: 50.8 mm (2 inches) diameter × 2.24 m (88 inches) long.
Air flowrate = 28.3 L/min (1 cfm).

[(2)] Tube 2: 63.5 mm (2.5 inches) diameter × 1.80 m (71 inches) long.
Air flowrate = 780 L/min (27.5 cfm).

2.2.2 Error Analysis.

The methodology for describing and analyzing uncertainties in single sample experiments is given by Kline and McClintock[16]. Application of their approach to the expression for concentration, Equation 6, reduces to,

$$\frac{e(c)}{c} = \pm\sqrt{\frac{e^2(R)}{R^2} + \frac{e^2(V_L)}{V_L^{\,2}} + \frac{e^2(q_A)}{q_A^{\,2}} + \frac{e^2(t)}{t^2}} \tag{7}$$

where: $e(x)$ denotes the estimated uncertainty in the parameter within the parenthesis. We assume that $x \pm e(x)$ represents the equivalent of an estimated 95% confidence interval.

The independent variables are fluorometer readings (R), solution volume (V_L), volumetric flowrate of air q_A, and time t. When the penetration of the aerosol is about 50%, we estimate the relative uncertainties (e.g., $\sqrt{e^2(R)/R^2}$) in these parameters to be 0.12, 0.02, 0.02, and 0.05, respectively. For the reading uncertainty, the value of 0.12 is about twice the average standard error of fluorescent measurements on the 1X and 10X scales; and the time uncertainty is large because it includes startup and shutdown of the blowers. Using these values in Equation 7, gives a relative uncertainty in concentration of $\pm$ 0.13. Applying the uncertainty principle to penetration, Equation 3, gives:

$$\frac{e(P)}{P} = \pm\sqrt{\frac{e^2(c_{dn})}{c_{dn}^2} + \frac{e^2(c_{up})}{c_{up}^2}} = \pm 0.19 \tag{8}$$

If the penetration were 50%, the uncertainty would be 50% $\pm$ 10% and that uncertainty is dominated by that of the fluorometer readings. The uncertainty analysis assumes the relative error bands to encompass the 95% confidence interval of the random variable, which is approximately $\pm$ two standard deviations.

3. DISCUSSION AND CONCLUSIONS

Any experimental study must have a solid quality assurance foundation, and in this study we have examined the parameters that influence analysis of aerosol tests where fluorescein is used as an analytical tracer. Several of the tests reproduce those performed earlier by others; however, we sought to not only verify those results, but to present them and new results in a single reference. Such a reference should provide a tool for training aerosol researchers in quality assurance aspects of fluorometry and it should provide a summary of factors that need to be considered in any aerosol study that employs fluorometric analyses.

Evaluation of the excitation and emission spectra for fluorescein show peaks at 492 nm and 516 nm, respectively. For fluorescein, a narrow band transmission optical filter (NB490), which has a nominal band width of 10 nm can be used on the excitation light and a sharp cut optical filter (SC515) can be used for the emission light. The latter filter has about 37% transmission at 515 nm, less than 1% transmission for 505 nm light, and about 80% transmission at a wavelength of 525 nm. A three-dimensional plot, which shows the intensity of the emission light as a function of the excitation and emission wavelengths, indicates there is a clearly defined maximum in intensity at an emission wavelength of about 516 nm over the range of excitation wavelengths from 440 nm to about 510 nm.

The pH of the solution can have a profound impact on the fluorescence intensity of a sample. The normalized intensity is essentially 100% if the pH value of a fluorescein solution is greater than 9, and less than about 1% if the pH is less than 5. For extraction and analysis of fluorescein, we use a solution of deionized/distilled water and 2-propanol (50/50 on a volume basis) with 0.056% (volume basis) of 14.8 N ammonium hydroxide, which has a pH of 9.8.

A set of tests was conducted to determine whether the fluorometer reading and fluorescein concentration are directly proportional. A statistical regression was performed on the logarithm of an expression of the form $R = a\,c^{b}$, where R is the fluorometer reading, c is the fluorescein concentration and a and b are constants. If the two are directly proportional, the value of b is unity. Over a range of fluorescein concentrations from 10^{-9} to 0.75×10^{-6} g/mL, with three instrument sensitivities (1X, 10X and 50X scales), the slopes range from 0.982 to 1.039. We consider this type of calibration to be important because it is not only a check on the instrument, but it can also provide a check on methodology as it is known on an *a priori* basis that the exponent, b, must be unity provided the concentration is less than a self-quenching limit. We selected the limit to be 0.75 µg/mL (readings on the 1X scale < 1000) because it provided a value of b that is 0.982. Inclusion of readings from 1000 to 2000 on scale 1X reduced the value of b to 0.953. If only readings between 10 and 200 are considered, the slope is 1.00, and if readings in the range of 500 and 1500 (average of 1000) are considered, the slope is 0.89; which suggests that over the range of 10 to 1000, the slope varies from 1 to 0.9.

The ratios of scale factors for the 10X/1X scales and the 50X/1X scales were also checked. The results showed that the actual ratio of 10X/1X was 9.48 rather than 10 and the ratio of the 50X/10X was 4.8 rather than 5. In an experimental program, if results are to be based on readings from two different scales, those scale factors should be determined experimentally. This could be done through a simple calibration procedure that may be no more complicated than taking readings of the same solutions on each of the two scales.

The fluorometer manufacturer suggests that tubes should be marked so that they can be oriented in the fluorometer in the same manner each time they are used. This implies that tubes would be re-used. Because we use a new tube for each solution, we checked the effect of orientation in the fluorometer and the results showed the precision of readings with randomly oriented tubes is comparable to that for aligned tubes for scale factors of 1X to 50X. Also, the number of times a tube needs to be thoroughly rinsed was checked in order to render it compatible with re-use, and for the washing procedure that was used, the residual concentration after the third wash was about 1% of the initial concentration.

Often aerosol tests involve collection of the tracer-tagged particulate matter on a filter and subsequent elution of the tracer for analysis. A test was conducted to determine the efficiency of fluorescein extraction from a glass fiber sampling filter, and the results showed the elution was quantitative. However, the approach that was used for extraction involves shaking the filter in a solution of water/propanol and base for 1 hour. If less severe agitation were employed in a test procedure, the extraction might not be as efficacious.

For some organic fluorescent compounds, there is a degradation of fluorescence with time, and because fluorescein aerosol samples may not be analyzed immediately after elution, the readings of several samples were checked over a twelve-day period. The samples, which were exposed to fluorescent laboratory lights during the day, showed no systematic loss of fluorescence.

The precision of the fluorometric approach was examined in two ways. First, tests were run to characterize the penetration of aerosols through two transport tubes. For 11 µm AD aerosol particles, the penetration through Tube 1 was 56% ± 1% (mean ± 1 standard deviation) and that through Tube 2 was 96% ± 4.5%. Tests with 5 µm AD aerosol particles showed penetration values greater than 90% for both tubes and standard deviations that were 7.3% and 13.2%. For 15 µm AD aerosol particles only one tube was tested and the penetration was 28.3% with a standard deviation of 4.3%. Second, we used the single experiment uncertainty principle to estimate the variation in penetration and the results showed that if the penetration is 50%, an error band that is approximately ± 1 standard deviation would be 50% ± 8%.

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